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Zoophthora radicans (Zygomycetes: Entomophthorales) conidia production from naturally infected *Empoasca kraemeri* and dry-formulated mycelium under laboratory and field conditions

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Abstract

Laboratory and field studies were conducted to assess the effects of temperature on sporulation of a dried-mycelium formulation of the entomophthoralean fungus *Zoophthora radicans* and to compare sporulation of laboratory-produced/formulated fungus versus fungus occurring on cadavers of naturally infected *Empoasca* leafhoppers. Conidia production by the formulation increased from 3.1×10^4 to a maximum of 13.7×10^4 conidia/mg (dry weight) over the temperature range from 5 to 20 °C and decreased to 10.7×10^4 conidia/mg at 25 °C and to nearly zero at 31 °C. A temperature-dependent development model estimated a sporulation optimum of 23.6 °C. Pieces of formulated mycelium ($2 \times 2 \times 0.5$ mm) placed on bean and cowpea foliage in the field showed a temporal pattern of nightly conidial discharge similar to the fungus on leafhopper cadavers; both fungi initiated sporulation within a few hours following dewset and ceased with the return of dry conditions after 08:00 h. However, sporulation of the fungus on cadavers peaked between 00:00 and 03:00 h, while peak sporulation of the formulated fungus usually occurred shortly after dawn. Fungus on adult leafhopper cadavers and the pieces of formulated fungus underwent multiple daytime desiccation/nighttime rehydration cycles, producing conidia for up to eight consecutive nights. Second-, third-, fourth-, and fifth-instar cadavers supported sporulation for only 5–6 nights. On a dry weight basis, the fungus on cadavers produced substantially more conidia than the formulated fungus; however, differences were less pronounced based on the surface area of the hymenium. In general, the dried-mycelium pieces generated conidia in a manner similar (both temporally and quantitatively) to the fungus on leafhopper cadavers. These results indicate that the dried-mycelium formulation is well suited as an inoculum source for initiation or augmentation of epizootics in leafhopper populations.

Keywords: *Zoophthora radicans*; Leafhopper; *Empoasca kraemeri*; Cicadellidae; Biological control; Mycelium formulation; Sporulation

1. Introduction

The entomophthoralean fungus, *Zoophthora radicans* (Brefeld) Batko (Zygomycetes), is a commonly occurring entomopathogen and important natural mortality factor in populations of many insect pests, especially lepidopteran and homopteran species. This fungus has been the subject of much research aimed at harnessing its high-natural epizootic potential for biological control (Dustan, 1927; Jaques and Patterson, 1962; Leite et al., 1996a; McGuire et al., 1987a,b; Milner et al., 1982; Pell and Wilding, 1994; Soper, 1985; Wraight et al., 1986). Success in the controlled introduction of this fungus into pest populations to induce field-wide epizootics or to augment enzootic disease levels within naturally infected populations, will require a thorough knowledge of pathogen biology and epizootiology and a reliable source of inoculum.

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Greatest success in commercialization of entomopathogenic fungi has been achieved with hyphomycete species (e.g., *Beauveria bassiana* (Balsamo) Vuillemin and *Metarhizium anisopliae* (Metschnikoff) Sorokin). These fungi produce dense masses of dry, hydrophobic conidia that are stable under a broad range of environmental conditions and are therefore easily mass produced and formulated for microbial control applications. The conidia of the entomophthoralean fungi, on the other hand, are thin-walled, mucilagenous propagules that are actively dispersed. Compared to hyphomycete conidia, these ballistoconidia are extremely difficult to mass produce, harvest, and formulate on an industrial scale. Thus, while the impressive epizootic potential of these pathogens continues to stimulate commercial development efforts, the problems with production, stabilization, and formulation remain serious constraints.

The difficulties with handling of entomophthoralean conidia have led researchers to pursue other development strategies. One of the most promising has involved production of mycelium-based formulations. A significant advance in this area of research was made in the mid 1980s when USDA-ARS researchers developed and patented a process for desiccation–stabilization of *Z. radicans* mycelium (McCabe and Soper, 1985). The process provides a readily manipulable granular formulation of *Z. radicans*, which upon rehydration, rapidly generates large numbers of infectious ballistoconidia. Experimental applications of this material have successfully established infection foci (Wraight et al., 1986) and induced or augmented high-level epizootics in pest populations (Wraight and Galaini-Wraight, unpubl. results in Wraight and Roberts, 1987; Leite, 1991).

Despite these promising results, commercial biopesticide products based on *Z. radicans* dried mycelium have not materialized. There are numerous reasons for this slow development; however, the most important relates to the mode of action of the formulation. The product, itself (dried mycelium), is not infectious. To be effective, the mycelium must rehydrate and produce conidia. These conidia may, in turn, produce secondary conidia before ultimately succeeding in infecting a host. This process can be remarkably efficient, but it is extraordinarily sensitive to environmental conditions, especially free moisture (Galaini-Wraight et al., 1991). Environmental conditions in the field are largely uncontrollable, and it is difficult to base a commercial enterprise on a product that is efficacious only under a narrow range of environmental conditions. This problem is exacerbated by the need for prolonged favorable conditions to promote epizootic development beyond the first wave of infection achieved by formulation-derived conidia. Costs of using the product for inundative microbial control applications would almost certainly be prohibitive (Wraight et al., 1986). Clearly, these problems reveal a great need for new technologies (e.g., humectant formulations) to promote further development.

Needed, also, is a sound knowledge of the sporulation requirements and capacity of the dried-mycelium formulation following foliar applications. The sporulation characteristics of this and similarly produced formulations of *Z. radicans* have been studied only to a limited degree under laboratory conditions (Li et al., 1993; Pell et al., 1998; Soper, 1985). In this paper we report results of laboratory and field studies which characterize and compare the conidiogenesis of *Z. radicans* mycelium from mass-produced dried mycelium and naturally infected leafhoppers as a function of time, temperature, moisture, and host size (instar), and discuss the implications with regards to *Z. radicans* epizootiology and leafhopper biological control potential.

2. Materials and methods

2.1. Fungal preparations

ARSEF isolate 789 of *Z. radicans* originated from a leafhopper, *Empoasca kraemeri* Ross and Moore (Cicadellidae), collected in 1982 at the Centro Nacional de Pesquisa de Arroz e Feijão (CNPAF) near Goiânia, Goiás, Brazil. ARSEF isolate 1590 was collected from the same host species and location in 1984. These two isolates were shown to have identical rapid amplified polymorphic DNA (RAPD) profiles by Hodge et al. (1995).

Mycelium of each isolate was mass produced according to a protocol developed by D. McCabe and R. Soper (unpublished). This process was described briefly by Soper (1985) and was adapted for production of hyphomycete mycelia (Pereira and Roberts, 1990; Rombach et al., 1986); however, no detailed description of the *Z. radicans* mass production system has been published and so is included here. The described process includes a number of minor modifications that we developed.

Samples of each isolate removed from liquid nitrogen storage were grown on Sabouraud dextrose agar supplemented with yeast extract (1%). A small block of agar (5-mm square) was cut from the growing edge of a colony and inoculated into a 250-ml flask containing 100 ml sterile Sabouraud dextrose broth, also with 1% yeast extract, and adjusted to pH 6.8. The flask was incubated on a rotary shaker (250 rpm) at room temperature (21–25 °C) until a thick growth of mycelium was obtained (usually 96 h). The combined biomass from replicated flasks served as inoculum for mass production.

Mycelia were mass produced in simple fermenters fabricated from 20-liter polypropylene, screw-cap carboys. A hole was drilled in the shoulder of each carboy

and fitted with a rubber stopper supporting a glass tube (4.8-mm inside diam.) that extended to the bottom of the carboy. A rubber tube was attached to the upper end of the tube and closed with a pinch clamp.

To the carboy was added 1 liter nonsterile, deionized water, 100 g industrial grade yeast extract, 100 g dextrose, and 29 ml of 2 M NaOH. The carboy was manually agitated to dissolve the ingredients and then autoclaved for 20 min. A second batch of nutrient concentrate was prepared in a large flask, autoclaved and set aside for later use. After autoclaving, an additional 9 liters nonsterile, deionized water was added to the carboy by attaching the rubber tube directly to the deionized water spigot and filling to a mark. The carboy was then placed in a clean hood, and 1 liter (10 flasks) of inoculum was added along with 10 ml sunflower oil (Wesson, ConAgra Grocery Products, Irvine, CA), 10^6 units penicillin, and 1.25 g dihydrostreptomycin. Working quickly, the inoculated carboy was set in a large plastic tub on the laboratory bench, and the rubber tube was connected to a compressed air supply fitted with two in-line filters (glass wool/activated charcoal followed by a membrane HEPA). The carboy cap was loosened, and airflow was initiated and adjusted to a rate that generated as strong a circular flow as possible without blowing liquid out around the cap (some leakage normally occurred, especially near harvest, and was contained by the plastic tub). The loose cap was taped in position. After 24 h incubation at room temperature, the carboy was disconnected from the air line, placed in the clean hood, and the second batch of nutrient concentrate was added along with sunflower oil and antibiotics at the aforementioned rates. The carboy was then quickly topped to the 20 liter mark with water and reconnected to the air source. After an additional 18 h incubation, the carboy was monitored closely so as to harvest at maximum productivity. This was identified as the point when the culture grew so thick that circulation and thus aeration was impeded (in most cases, ca. 48 h postinoculation).

The mycelium was harvested, processed, and dried according to an elaborate protocol developed by McCabe and Soper (1985); a brief description follows. The whole culture was harvested by pouring through a coarse sieve (18 mesh). The mass of mycelium collected in the sieve was rinsed under a stream of nonsterile, deionized water, and then resuspended in an equal volume of water. The resulting slurry was filtered through a sheet of canvas fabric on a vacuum table (1 liter slurry/30 cm^2) to obtain a thin mat. The mats were peeled from the filter, placed on racks, and set out on laboratory benches to dry for 2 h at room temperature (21–25 °C). The mats were then sprayed to saturation with a solution of maltose (10%) and incubated for an additional 4–5 h. After this time, the mycelium mats (with well developed hymenia) were stacked in a 4 °C incubator and left for 18 h. Finally, the mats were removed from refrigeration and dried rapidly in the draft of a laboratory fume hood. The mats dried to crispness in approximately 6 h. Moisture content of the dried mycelium used in the studies reported here was not determined; however, all production was undertaken during winter or early spring in Ithaca, NY, when relative humidity in the laboratory was usually <30%. Approximately 130 g dry mycelium was obtained from each fermenter (6.5 g/liter of liquid medium). The dried mycelium was stored in sealed plastic bags at −20 °C until use. The dried mycelium of isolate 789 used in the laboratory sporulation experiment (conducted September 1983) was produced in March 1983. The dried-mycelium preparations of isolates 789 and 1590 used in all other studies were produced in February 1985 and April 1986, respectively. For the laboratory experiment, mycelium was ground into small granules using a mortar and pestle. Granules used were those passed by a 1-mm sieve and retained by a 0.5-mm sieve. For the field tests, the dried mycelium (ca. 0.5-mm thick) was cut into small square pieces (2 × 2 mm).

2.2. Laboratory studies

Twenty-five milligrams of dried-mycelium granules were spread evenly onto an agar substrate (1.5% agar containing antibiotics as described above) in the bottom of a 60-mm diameter plastic petri dish. The dish with fungus was inverted over a matching dish containing 1.5 ml of a conidia killing/preserving solution containing 1% Triton X-100 and 0.2% maleic acid (Soper, 1985). Five replicate dishes were placed in each of 6 unlighted incubators providing temperatures of 5, 10, 15, 20, 25, and 31 ± 1 °C. Every 2 h during the first 70 h of incubation and at 6-h intervals thereafter, the conidia-collecting dishes were replaced with dishes of fresh preservative. The conidia suspension from each collection dish was transferred to a small polypropylene test tube and stored at 4 °C.

Using the inoculation methods described by Wraight et al. (1990), 75 early fifth-instar nymphs of *E. kraemeri* were inoculated with conidia of *Z. radicans* isolate 1590 at a rate of ca. 30 conidia/mm^2 and incubated at 20 °C. The inoculations were conducted at CNPAF using leafhoppers from a laboratory colony derived from insects collected in 1987 from CNPAF research fields and maintained on beans (*Phaseolus vulgaris* L.). Sixty-three nymphs succumbed to fungal infection within 4 days. The cadavers were collected before fungal outgrowth and sporulation, dried over silica gel for 48 h, and weighed. Sample pieces of the dried-mycelium formulation were also weighed.

2.3. Field studies

Black beans, *P. vulgaris* var. Rio Tibagi were planted on a small research plot (ca. 60 × 20 m) at CNPAF on

21 March 1985. Rows were spaced 0.67 m. Furrow irrigation was applied at 3–4 day intervals during the study (15 May–7 June). Growth of the plants was limited on the site; the rows remained open and the canopy sparse. Cowpea *Vigna unguiculata* (L.) Walp. was planted on 30 March, 1987 at CNPAF in a plot of similar size. The last significant rainfall of the rainy season was recorded 7 May, and overhead irrigation was applied at approximately weekly intervals starting 21 May but was not applied during sporulation monitoring. By the time the experiments were initiated (12 May), the cowpeas had nearly filled the rows and produced a dense canopy. Both field sites were heavily infested with *E. kraemeri*, and natural epizootics of *Z. radicans* were in various stages of development (Galaini-Wraight et al., 1991).

2.3.1. Field experiment one (Phaseolus, May 1985)

At 1830 h on the evening of 15 May, 1985, ca. 200 pieces of dried mycelium of *Z. radicans* isolate 789 were placed on moist filter paper and transported to the field. Using forceps, the pieces were applied to the undersides of upper-canopy bean leaves (one piece per leaf). Each piece was situated so that one side contacted the leaf midrib. The pieces adhered readily to the dew-covered leaf surface. Small conidia traps were then placed over the mycelium pieces to capture the actively discharged conidia. The traps were fabricated from Fisherbrand 22-mm-square plastic microscope coverslips (Fisher Scientific, Pittsburgh, PA). These were cut in half, folded at three points, and mounted on insect pins (Fig. 1). Using a fine paintbrush, the conidia-collecting surface of each trap was coated with a gelatin solution (gelatin at 10% dissolved in the previously described conidia poison/preservation solution heated to 45 °C) and allowed to dry. One trap was placed over each piece of mycelium, being attached by pushing the pin through the leaf and into a 1-cm foam cube. The gelatin-based substrate inhibited conidia germination and secondary-conidia production and allowed easy removal of dense conidial deposits for enumeration (see below).

The application of the fungus and conidia traps required ca. 1 h and 45 min. During this time, three fresh *Z. radicans*-killed *E. kraemeri* (2 late-instar nymphs and 1 adult) were discovered on the undersides of bean leaves and were also covered with traps. At 22:00 h and at each hour thereafter until 11:00 h the next morning, 10 randomly selected traps were collected. In this way, traps collected at 22:00 h contained all conidia produced from the time of rehydration of the mycelium (18:00 h) until 22:00 h, traps collected at 23:00 h contained all conidia produced between 18:00 and 23:00 h, and so forth. Hourly estimates of conidia production were then obtained by subtraction of the cumulative means. A group of 40 traps was left undisturbed the entire night to estimate total conidia production per mycelium piece. The traps on the leafhopper cadavers were collected on the same hourly schedule; however, in this case each collected trap was immediately replaced with a clean trap.

Preliminary observations in the field revealed that fungal sporulation on leafhopper cadavers ceased with the return of low-humidity conditions after dawn, but rehydration of the fungus on subsequent nights resulted in additional sporulation (Galaini-Wraight et al., 1991). The mycelium pieces exhibited a similar cycle of sporulation–desiccation dormancy, and we continued to monitor conidia production after night one.

On the second night (16–17 May), conidia traps were placed over the same mycelium pieces and leafhopper cadavers monitored the first night, and the traps were collected exactly as on night one (traps were collected from the same formulation pieces at the same times each night). On subsequent nights, traps were placed over 40 randomly selected mycelium pieces and the leafhopper cadavers and were left in place the entire night to estimate total nightly conidia production. The same 40 formulation pieces were monitored each night until the end of the experiment. Trap samples derived from mycelium pieces or leafhopper cadavers with fungus that had ceased to produce conidia were included in mean calculations, while samples from lost mycelium pieces or cadavers were omitted. Mycelium pieces were occasionally removed by ants, bees, and other insects apparently attracted to the sugar used as a drying protectant in the formulation process.

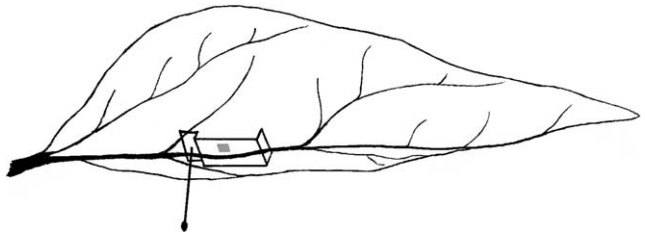

Fig. 1. Diagram of conidia trap positioned over a piece of sporulating mycelium.

2.3.2. Field experiment two (Phaseolus, May 1985)

At 15:00 h on 19 May, a search was initiated to locate moribund leafhoppers naturally infected with *Z. radicans*. Approximately 20 bean leaves with immobile, dying leafhoppers (5 second-, 4 third-, and 9 fifth-instar nymphs and 1 adult) were flagged. At 19:00 h, conidia traps were placed over the cadavers, which were all located on the undersides of leaves. At the same time, five pieces of mycelium were also placed on the bean foliage as previously described. Commencing at 21:00 h, and continuing at hourly intervals through the night, the

conidia traps were collected and replaced with clean traps. On the following night, the hourly sampling procedure was repeated. On subsequent nights, total nightly conidia production was determined by placement of a single trap over each cadaver or formulation piece and retrieving all traps the following day (after cessation of sporulation).

2.3.3. Field experiment three (Phaseolus, June 1985)

The mycelium sporulation experiment described above (experiment 1) was repeated 3 weeks later (commencing 5 June). In addition to the collection of conidia traps from a different group of 10 mycelium pieces at each of various times during the night, 50 pieces were left in place each night to determine total conidia production per mycelium piece. No leafhopper cadavers were monitored.

2.3.4. Field experiments four and five (Vigna, May and June 1987)

Further experiments, similar to the 1985 studies, were conducted in 1987 in cowpea using a dried-mycelium formulation of isolate 1590. In experiment four, 25 hydrated mycelium pieces were placed on the foliage at 18:30 h on the evening of 12 May, and total conidia production was monitored for the following 9 nights. No hourly monitoring of sporulation was undertaken.

Experiment five examined the host-instar-dependent sporulation of *Z. radicans* using essentially the same procedure as experiment 2. Twenty-six naturally fungus-killed leafhoppers of mixed instars were monitored; sporulation on these cadavers was also compared to sporulation from six pieces of isolate 1590 mycelium.

2.4. Conidia counting

Conidia collected in the laboratory study were counted as follows. One gram glass beads (0.5-mm diam.) was added to each sample tube, and a uniform suspension of conidia was created by vortexing for 15 s. The tube was set aside to allow for dissipation of foam. The conidia were resuspended by several short bursts on the vortex mixer and poured rapidly onto the surface of a thin layer of gelatin (10%) in a 35-mm diam. petri dish lid and allowed to settle for 10–15 min. The gelatin constituted a sticky substrate to which the settling conidia adhered lightly, permitting handling of the dish with minimal disruption of the uniform pattern of conidia deposition. The dish was placed on a microscope stage and scanned from edge to center (along the dish radius) with a 10× objective. All conidia passing between selected points on an eyepiece micrometer scale positioned at right angles to the scanning direction were counted. The points on the micrometer were selected in accordance with the number of conidia in the sample; e.g., the entire width of the micrometer scale was used in counting samples with few conidia while only 5% of the width was used for high-density depositions. The maximum/minimum area (swath) counted was 17.55/0.88 mm^2. After the first count, the dish was rotated 90° and a second scan was counted. If the counts from the two scans were markedly different (coefficient of variation >50%), the dish was rotated an additional 45° and a third scan was made. The mean of the counts was then multiplied by a factor accounting for the entire dish area.

The conidia traps collected during the field experiments were stored dry at room temperature. Counting of most samples was accomplished by peeling the dried film of gelatin supporting the conidia from the trap and dropping it into a small test tube containing 1.5 ml of a 0.1% Tween solution. The solution was warmed to 45 °C to melt the gelatin and release the conidia, and conidial aggregates were dispersed using a vortex mixer. Conidia counts were made using the methods described above. In cases where very few conidia were deposited, the conidia traps were observed directly under the microscope (50×) and total conidia were counted.

2.5. Environmental monitoring

Thoughout the field experiments, environmental conditions, including leaf wetness and temperature, were monitored at canopy level using electronic data loggers (Omnidata International, Logan, UT). Hours of wet conditions reported in the tables are the cumulative times during which the leafwetness sensor was absorbing moisture from the air (sensor readings increasing) or indicating stable wet conditions. Intervals during which the sensor readings decreased were considered as time under drying conditions (see Galaini-Wraight et al., 1991). The leaf wetness sensor gave a reading of 6 U when completely dry. The time of evening marking the beginning of moisture readings above six are considered as the time of initiation of dewset, even though droplets of dew usually became visible to the unaided eye at a reading of approximately 40 U. The fully saturated probe gave a reading of 225.

2.6. Statistical analyses

Peak sporulation rates at each temperature (conidia/h/g mycelium) were estimated by fitting the observed hourly sporulation rates to the log normal distribution. The peak rates were regressed on temperature using the Sharpe and DeMichele temperature-dependent development model (Sharpe and DeMichele, 1977; Wagner et al., 1984; computer program written by T. Larkin [unpublished]). Conidial numbers were log transformed and compared by ANOVA using the JMP statistical software (SAS Institute, 1995).

3. Results

3.1. Laboratory sporulation of dry-formulated mycelium

The temporal patterns of conidia production by the formulated mycelium granules incubated under constant temperature conditions were fitted to the log normal distribution (Fig. 2). Significant deviations from the models are evident at each temperature. Lack of fit was attributable, in large part, to heterogeneity about the model resulting from sporulation pulses. However, a more systematic departure from the model was manifest as a lower than expected sporulation rate beginning approximately 20 h posthydration and lasting for 8–10 h or longer. This was especially evident at 5 and 10 °C. At these temperatures, the fungus also produced conidia at greater postpeak rates than predicted by the model. These results suggest that a different model might provide a better estimate of low-temperature sporulation. Nevertheless, an underlying log normal distribution of conidia production is strongly suggested by our data and by data from other studies of Entomophthorales (Milner, 1981), and the model was accepted as an adequate predictor of the sporulation process.

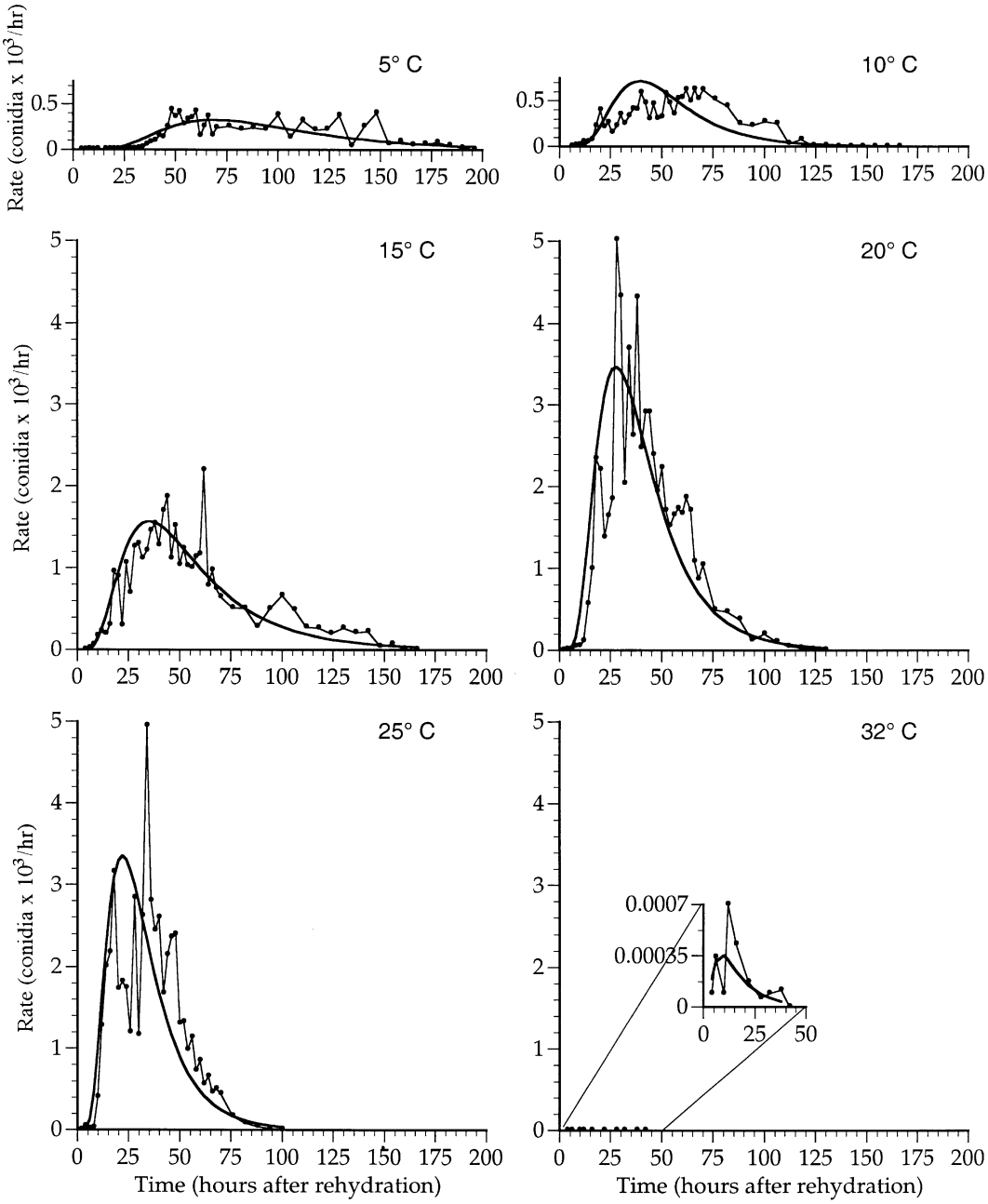

Fig. 2. Rates of conidial production (conidia $\times 10^3$/h) per mg (dry weight) of formulated mycelium of *Z. radicans* strain 789 incubated under constant temperature conditions in a laboratory test fit to the log normal distribution.

Conidia production was greatly influenced by temperature over the range of 5–31 °C (Table 1). The greatest number of conidia was produced at 20 °C; sporulation was almost completely inhibited at 31 °C. Conidia were detected in the samples within 20 h at 5 °C, 8 h at 10 °C, and 4 h at 15–31 °C. The time required for initial conidia production estimated by the models (arbitrarily considered the time from hydration to production of 1% of the total conidia) showed an even stronger inverse relationship to temperature (Table 1). Similar inverse relationships were observed for duration of sporulation (time to 99% of total conidia production), median sporulation time, and the time to peak sporulation (Table 1). All correlations were statistically significant. The analysis of the temperature-dependent, peak rates of sporulation indicated an optimum temperature for sporulation of 23.6 °C (Fig. 3).

The median sporulation times were transformed to rates (h^{-1}) and regressed on temperature (Fig. 4). The model estimated a base temperature (sporulation threshold) of 1.78 °C and thermal constant of 510.2 degree-hours (DH).

3.2. Field experiment one

In the following presentations, the laboratory-produced fungus will be referred to as the formulated fungus (or mycelium formulation), and the fungus on the leafhoppers will be referred to as the fungus on cadavers or naturally occurring fungus.

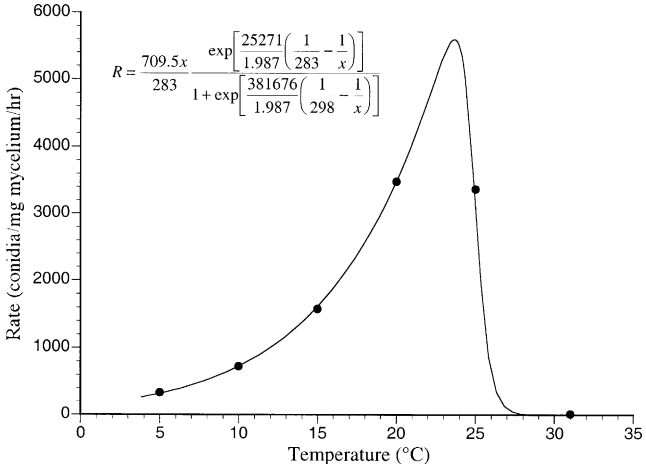

Fig. 3. Temperature-dependent peak rates of sporulation of formulated mycelium of *Z. radicans* (estimates from Fig. 1) fit to the Sharpe and DeMichele temperature-dependent development model, where R, sporulation rate (conidia/mg mycelium/h) and x, temperature in K.

Dewset commenced at 19:00 h on the first night of the experiment (15–16 May), and conidia were detected from two of the three leafhopper cadavers by 22:00 h and from all three by 23:00 h. Traps were collected from 10 different (randomly selected) formulation pieces each hour during the night. Conidia were detected on four of the 10 traps collected at 22:00 h and on all 10 traps collected at 01:00 h. Temperature declined gradually from 22 °C at 18:00 h to 15 °C at 06:00 h. Conidial discharge by both the formulated fungus and fungus on cadavers terminated by 09:00 h, coinciding with the postdawn

Table 1
Temperature-dependent sporulation of a dried-mycelium formulation of *Z. radicans* isolate 789 rehydrated and incubated under laboratory conditions

Temperature (±1 °C)	Total conidia $\times 10^3$/mg[a]	Linear model[b]	Observed hours until 50% sporulation	Model-predicted hours and degree hours[c] until			
				1% sporulation	Peak sporulation	50% sporulation	99% sporulation
5	31.2 ± 4.60	$Y = -4.12 + 2.04 (\ln X)$	96.4	28 (90)	68.8	87.5 (280)	274 (877)
10	34.6 ± 2.28	$Y = -3.69 + 2.24 (\ln X)$	61.3	17 (139)	39.4	48 (394)	135.5 (1111)
15	91.4 ± 15.44	$Y = -1.88 + 1.78 (\ln X)$	52.7	13 (172)	35.2	48.3 (638)	197.2 (2365)
20	137.2 ± 22.54	$Y = -2.12 + 1.99 (\ln X)$	38.8	11.1 (202)	27.7	35.7 (650)	114.7 (2088)
25	106.8 ± 14.98	$Y = -1.61 + 1.97 (\ln X)$	33.8	8.8 (204)	22	28.5 (661)	92.5 (2146)
31	0.006 ± 0.0002	$Y = 1.18 + 1.44 (\ln X)$	15.2	2.8 (82)	8.7	14.1 (412)	70.8 (2067)
Correlation coefficient (r)			−0.962 ($P < 0.005$)	−0.953 ($P < 0.005$)	−0.946 ($P < 0.005$)	−0.934 ($P < 0.01$)	−0.874 ($P < 0.05$)

[a] Observed total conidia $\times 10^3$ produced per mg mycelium (dry weight) ± standard error ($n = 5$).

[b] Lognormal distributions linearized by transforming to cumulative lognormal curves and applying the probit and logarithmic transformations; Y, probit cumulative proportion total sporulation per hour; X, ln hours after rehydation.

[c] Number in parentheses indicates degree hours (base temperature = 1.8 °C).

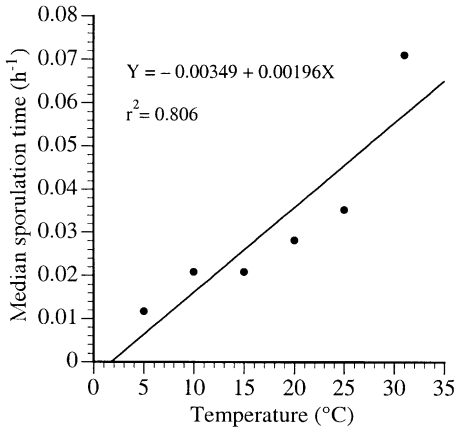

Fig. 4. Temperature-dependent rates of sporulation of formulated mycelium of *Z. radicans* fit to a linear model (rates expressed as inverse of the median sporulation times).

return of dry conditions. The sporulation profile with temperature and moisture data is shown in Figs. 5a–c.

On the second night, dew formation started at 20:30 h, and sporulation was detected by 21:00 h on two of the thee leafhopper cadavers and on seven of nine mycelium pieces (one piece from the sample group was missing). Sporulation began on all cadavers by 22:00 h and all formulation pieces by 00:00 h. Temperature declined from 22 °C at 19:00 h to 16 °C at 06:00 h. Conidial production ceased by 09:00 h (Figs. 5d–f).

The times of initiation and termination of sporulation were similar for the formulated fungus and fungus on cadavers, but the overnight patterns of sporulation differed markedly. The rate of sporulation of fungus on cadavers peaked by 02:30 h on both nights, whereas sporulation of the formulation peaked shortly after dawn. Although changes in moisture conditions were clearly associated with initiation and termination of sporulation, the major and minor pulses of sporulation recorded during the night were not consistently associated with fluctuations in moisture levels. Over some time intervals, sporulation rates declined sharply while moisture was increasing or stabilized at a high level. Although the postdawn increases in conidial production evident in three of the four sporulation curves of Fig. 5 were coincident with increasing temperature, the relationship between temperature and sporulation rate was also inconsistent. During some periods, opposite trends in sporulation of the formulated fungus and fungus on cadavers were observed.

Conidia production by both the formulated and naturally occurring fungi occurred during each of five consecutive nights (Table 2). Monitoring ceased after a severe storm at 18:00 h on 21 May removed much of the fungus from the bean leaves and the conidia from the traps set out the previous night that had not yet been collected. Until that time, persistence of the formulation pieces on the foliage had been excellent (93%). During the five nights, the cumulative total numbers of conidia produced were 1.54×10^5/formulation piece and 1.35×10^5/leafhopper cadaver. Although conidia production continued for at least five nights, sporulation

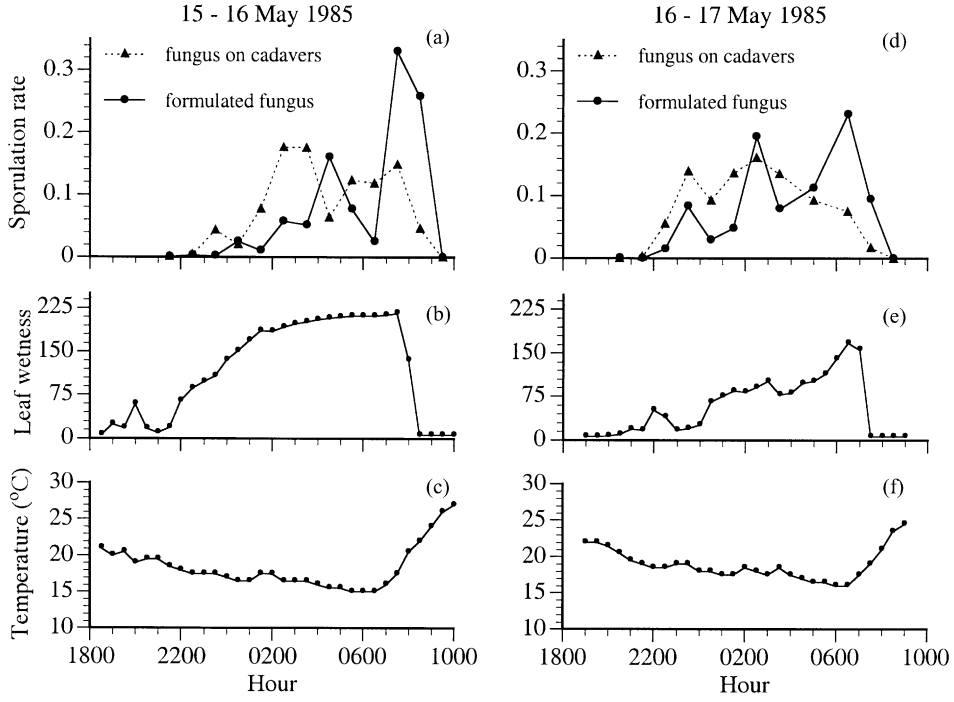

Fig. 5. Patterns of *Z. radicans* sporulation from formulated mycelium versus fungus on naturally infected leafhopper cadavers over two consecutive nights with temperature and leaf wetness data; sporulation rate expressed as proportion of total conidia/h.

Table 2
Zoophthora radicans conidia production from dried-mycelium formulations versus naturally infected *E. kraemeri* on bean and cowpea foliage in fields near Goiânia, Goiás, Brazil, 1985

Night of	Formulation pieces sporulated/monitored	Conidia $\times 10^3$ per formulation piece[a]	Cadavers sporulated/ monitored[b]	Conidia $\times 10^3$ per cadaver	Temperature (°C) mean (range) while foliage wet	Hours foliage wet	Degree hours under wet conditions
Field experiment one (*Z. radicans* isolate 789 on beans, 1985)							
15–16 May	40/40	40.3 ± 2.7	3/3	59 ± 18.2	16.8 (15–20)	11.5	173
16–17 May	40/40	47.4 ± 2.7	3/3	60.9 ± 8.4	18 (16–23)	8	130
17–18 May	38/40	15.7 ± 2.8	3/3	3.9 ± 1.3	15.4 (14–18.5)	8	109
18–19 May	39/40	34.7 ± 4.5	2/2	$10.8 \pm .07$	17.6 (16–21)	9	142
19–20 May	35/37	19.8 ± 3.5	1/1	0.5	18 (16.5–20.5)	9	146
Total		157.9 154.1 ± 9.85 ($n = 37$)[c]		135.1 117 ± 33.59 ($n = 2$)[c]			
Field experiment three (*Z. radicans* isolate 789 on beans, 1985)							
5–6 June	39/50	0.18 ± 0.04	—	—	8.4 (4.5–14)	14	92
6–7 June	16/50	0.03 ± 0.01	—	—	7.3 (4–11.5)	12.5	69
Total		0.21					
Field experiment four (*Z. radicans* isolate 1590 on cowpeas, 1987)							
12–13 May	16/25	0.4 ± 0.3	—	—	16.5 (14.5–19)	10.5	154
13–14 May	23/24	17.9 ± 5.7	—	—	17.3 (16–21)	8	124
14–15 May	23/24	26.6 ± 6.1	—	—	16.4 (15.5–18.5)	8	117
15–16 May	22/23	30.9 ± 6.1	—	—	18.3 (17–20)	9	149
16–17 May	22/23	38.5 ± 5.6	—	—	19.4 (17.5–24)	11.5	202
17–18 May	19/22	18.8 ± 5.1	—	—	19.3 (17–23.5)	10.5	184
18–19 May	14/21	1.8 ± 1	—	—	18.3 (16–21.5)	9.5	157
19–20 May	6/21	0.03 ± 0.02	—	—	17.6 (16–21)	8	126
20–21 May	0/21	0	—	—	17 (15.5–19)	7	106
Total Total from persistent substrates[d]		134.9 132.9 ± 10.42 ($n = 21$)					

[a] Mean number of conidia $\times 10^3$ produced per piece ($2 \times 2 \times 0.5$ mm) of dried-mycelium formulation ± standard error.
[b] Conidia collections were made from one fourth-instar, one fifth-instar, and one adult *E. kraemeri* cadavers.
[c] Total conidia $\times 10^3$ (mean ± standard error) produced from formulation pieces or leafhopper cadavers that remained attached to the bean foliage for five days.
[d] Total conidia $\times 10^3$ (mean ± standard error) produced by formulation pieces that remained attached to the cowpea foliage until completion of the sporulation cycle.

was most intense during the first two nights, amounting to 56 and 89% of the total 5-day production by the formulated fungus versus fungus on cadavers, respectively. This high level of sporulation occurred within only 303 DH (the total DH under moist conditions during nights one and two), a number substantially lower than the mean of 510 DH requirement for 50% sporulation predicted by the laboratory data. Significantly lower levels of sporulation on night three were associated with cool conditions.

3.3. Field experiment two

Dewset commenced at 18:00 h on the first night and earliest sporulation was observed by 21:00 h on four of the 19 cadavers and two of five mycelium pieces. Sporulation was initiated on 90% of cadavers by 01:00 h and all formulation pieces by midnight. Dewset was detected at 19:00 h on the second night, and sporulation was recorded on 65% of cadavers and 60% of formulation pieces by 21:00 h. Conidial production was observed on all formulation pieces by 22:00 h and on 94% of cadavers by midnight. The temporal patterns of sporulation on the different host instars were similar (Figs. 6a and e). The pattern of sporulation from all cadavers combined (mean sporulation rates) differed from that of the formulation (Figs. 6b and f). As observed in the first field experiment, the peak rate of conidial production by the fungus on cadavers occurred during the early morning hours (00:30–02:30 h), while peak sporulation of the formulated mycelium was recorded after dawn. Also, as noted in the first experiment, initiation and termination of sporulation were closely correlated with evening dewset and morning desiccation, respectively, whereas

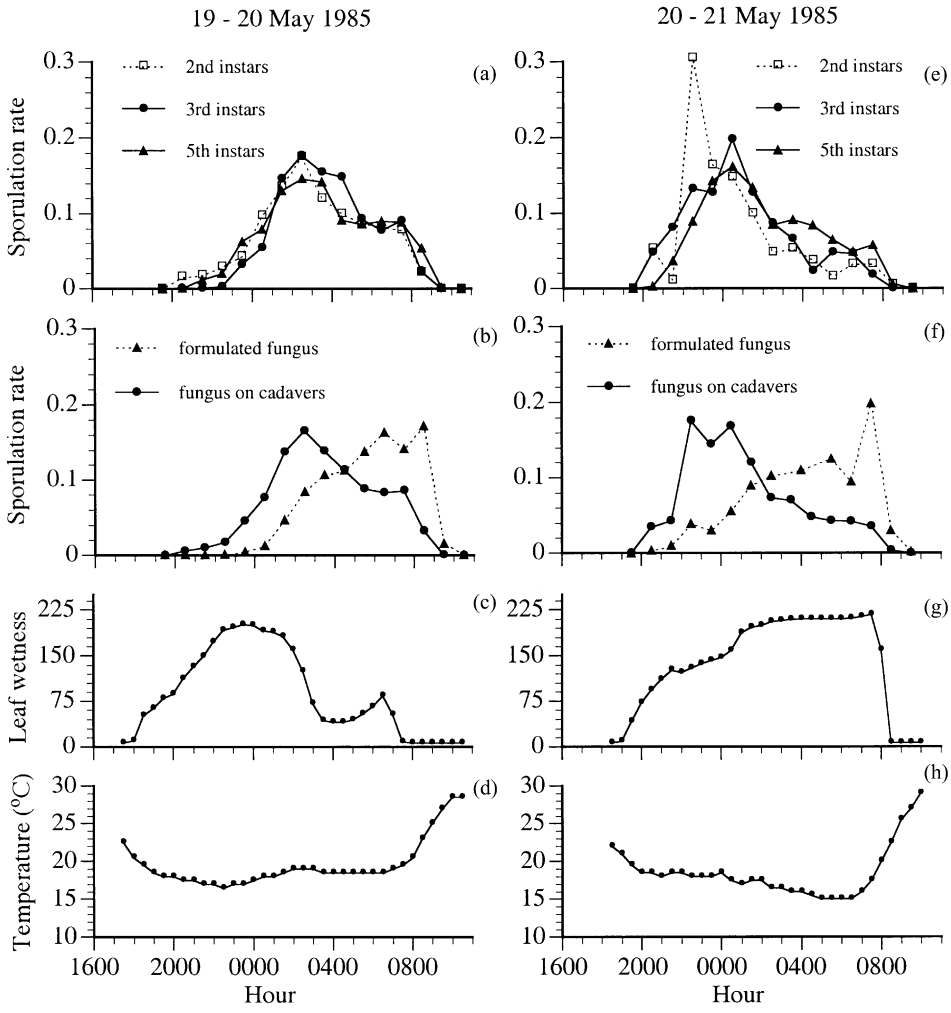

Fig. 6. (a and e) Patterns of *Z. radicans* sporulation from naturally infected second-, third-, and fifth-instar leafhopper cadavers over two consecutive nights with temperature and leaf wetness data; (b and f) patterns of sporulation from formulated mycelium versus fungus on naturally infected leafhopper cadavers (mean pattern from all instars); rate expressed as proportion of total conidia/h.

trends in sporulation appeared to develop independently of moisture levels during the night.

Leafhopper cadavers of the second- and third-instars supported sporulation on three consecutive nights, fifth-instars on four nights, and the formulation pieces on five nights (Table 3). Conidia production from the second- and third-instars was largely completed within the first two nights, from the fifth-instars within three nights, and from the mycelium pieces within four nights. Greater than 50% of total conidia production from the cadavers occurred during the first night (within only 145 DH). Conidia production from the formulation pieces exceeded 50% within the first two nights and 338 DH. Nine of 18 leafhopper cadavers and one of five formulation pieces were lost during the 21 May storm. The multiple sporulation cycles on second-, third-, and fifth-instar nymphal cadavers, adult cadavers, and mycelium pieces produced mean totals of 1.1, 1.9, 9.9, 15.9, and 33.7 conidia $\times 10^4$, respectively.

3.4. Field experiment three

Mean temperatures during wet conditions over the first two nights of this experiment were unusually low (only 8.4 and 7.3 °C, respectively), and conidia production was minimal (Table 2). Despite the cold temperatures, the pattern of sporulation of the *Z. radicans* isolate 790 formulation over the first night was similar to that described for the first experiment, with the peak rate of sporulation occurring after dawn (Fig. 7a). On the second night, during which only 32% of mycelium pieces sporulated and a mean of only 30 conidia were produced per formulation piece, sporulation peaked before midnight. However, a secondary peak of nearly equal magnitude occurred after dawn (Fig. 7b). Extremely cold temperatures persisted during the next several nights (ultimately dropping below 0 °C), and the experiment was terminated.

Table 3
Zoophthora radicans conidia production from a dried-mycelium formulation versus naturally infected *E. kraemeri* on bean foliage in a field near Goiânia, Goiás, Brazil, 1985

Night of	Conidia $\times 10^3$ produced per leafhopper cadaver or formulation piece (number of substrates sporulated/monitored)					Temperature (°C) mean (range) while foliage wet	Hours foliage wet	Degree hours under wet conditions
	Second-instars	Third-instars	Fifth-instars	Adults	Formulation pieces			
19–20 May	10.4 ± 1.4 (5/5)	16.3 ± 2 (4/4)	61.6 ± 7.9 (9/9)	103.2 (1/1)	115.3 ± 10.4 (5/5)	18 (16.5–20.5)	9	145
20–21 May	0.8 ± 0.4 (4/4)	2.4 ± 1.1 (4/4)	35.1 ± 8.1 (9/9)	56 (1/1)	92.5 ± 19.1 (5/5)	17.2 (15–21)	12.5	193
21–22 May[a]	0.02 ± 0.02 (1/2)	0.10 (1/1)	1.9 ± 0.8 (6/6)	—	113.5 ± 22.6 (4/4)	18 (16.5–20.5)	10.5	170
22–23 May	0 (0/2)	0 (0/1)	0.02 ± 0.01 (3/5)	—	15.4 ± 6.5 (4/4)	16.2 (14–21)	13	187
23–24 May	0 (0/2)	0 (0/1)	0 (0/5)	—	0.4 ± 0.1 (2/4)	16.5 (14.5–20)	14.5	213
24–25 May	—	—	0 (0/5)	—	0 (0/4)	17.9 (16–22)	11.5	185
Total	11.2	18.8	98.6	159.2	337.1			
Total from persistent substrates[b]	11.2 ± 2.10 ($n = 4$)	18.8 ± 2.18 ($n = 4$)	99.7 ± 10.33 ($n = 6$)	—	371.8 ± 38.82 ($n = 4$)			

[a] Nine of 18 cadavers were lost during a severe rain storm that occurred 18:00 h on 21 May.
[b] Total conidia $\times 10^3$ (mean ± standard error) produced from leafhopper cadavers or formulation pieces that remained attached to the leaf substrate until completion of the sporulation cycle.

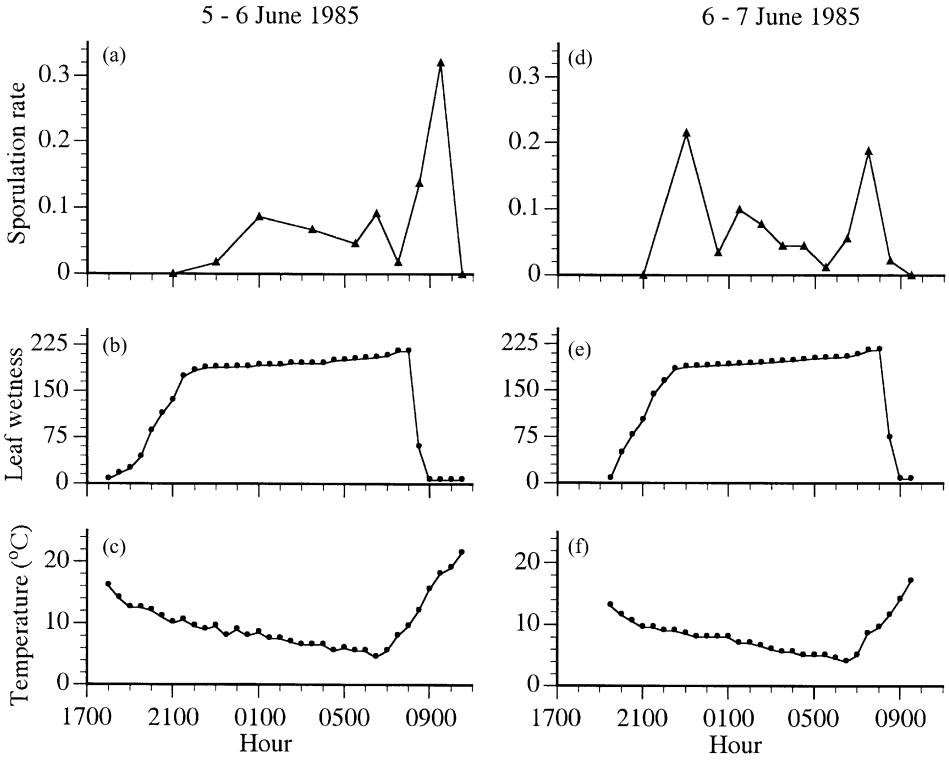

Fig. 7. Patterns of *Z. radicans* sporulation from laboratory-produced mycelium over two consecutive nights with temperature and leaf wetness data; sporulation rate expressed as proportion of total conidia/h.

3.5. Field experiment four

Following application of isolate 1590 mycelium, sporulation was recorded over eight consecutive nights (Table 2). Production totaled 1.35×10^5 conidia/mycelium piece. Sporulation was low the first night (0.4×10^3 conidia/piece) and increased to a peak of 38.5×10^3 on the night of 16–17 May, the night during which the maximum mean temperature (19.4 °C) and DH (202) were recorded. Sporulation exceeded half (56%) of the

total within four nights. The total DH under wet conditions over this time period was 544, similar to the 520 DH predicted for 56% sporulation of isolate 789 mycelium in the laboratory study. Persistence of the mycelium pieces on the cowpea foliage was 84% after 8 days. Mycelium pieces that ceased sporulation were rapidly overgrown with saprophytic molds.

3.6. Field experiment five

Conditions were cool during the experiment, especially during the first three nights when temperatures dropped to 9–10 °C, and the grand mean nightly temperature was only 12.5 °C (Table 4). Nightly degree hour accumulations were generally high, however, as a result of long dew periods (13.5–14.5 h). Dewset commenced at 19:00 h on the first night, but earliest sporulation was not observed until ca. midnight on one of six third-instar, one of six fifth-instar, and one of six adult leafhopper cadavers (Figs. 8a–d, Table 4). The fungus sporulated on 35% of the 26 total cadavers by 03:00 h, on 50% by 05:30 h, and on 73% by 09:00 h. Only one of the six pieces of formulation produced conidia during the first night.

Initiation of dewset was detected at 17:30 h on the second night, and earliest sporulation was observed by 21:30 h on two cadavers (both fourth-instars). By midnight, sporulation commenced on 29% of 24 remaining cadavers, on 67% by 03:00 h, and on 92% by dawn. Five formulation pieces remained and were monitored the second night; one initiated sporulation by 01:00 h and two by 05:30 h (Fig. 8f, Table 4).

As observed in the 1985 experiment, the temporal patterns of sporulation on the different host instars were similar (Figs. 8a and e). On the first night postapplication, the mean pattern of sporulation from all cadavers differed from that of the formulation (Fig. 8b). As observed in 1985, sporulation of the naturally occurring fungus peaked during early morning (02:30–03:00 h), whereas peak sporulation of the formulated mycelium was recorded after dawn. During night two, however, the two fungi exhibited similar patterns of conidial discharge with coincident peaks at 02:30 h and after dawn (Fig. 8f). Again it was evident that initiation and termination of sporulation were closely correlated with evening dewset and morning desiccation, respectively, whereas trends in sporulation developed independently of moisture levels during the night.

Second–fifth-instar leafhopper cadavers supported sporulation on 5–6 nights, whereas adult cadavers and formulation pieces supported conidia production for 7–8 nights (Table 4). Approximately half (44%) of total conidial production from all cadavers occurred during the first three nights and a total of 458 DH. Conidial production from the formulation pieces reached 46% after four nights and 640 DH (Table 4). After seven nights, 69% of the original 26 cadavers and 50% of the six formulation pieces remained on the cowpea leaves. The multiple sporulation cycles on first-, second-, third-, fourth-, and fifth-instar nymphal cadavers and adult cadavers produced mean totals of 0.2, 1.2, 2.1, 5.3, 11.7, and 27.4, conidia $\times 10^4$, respectively. A total of 6.9×10^4 conidia were produced per formulation piece.

3.7. Comparisons of adjusted sporulation values

The dry weight of the 63 fifth-instar nymphs killed by *Z. radicans* under laboratory conditions was 7.16 mg (0.114 mg/cadaver). The 2×2-mm dry mycelium pieces of isolates 789 and 1590 weighed an average (±SE) of 1.29 ± 0.048 and 1.23 ± 0.087 mg ($n = 10$), respectively. The surface area of the hymenium on fifth-instar cadavers and mycelium pieces was ca. 2 and 6.25 mm^2, respectively. These values were used as the bases for comparing sporulation of the fungus on leafhopper cadavers with sporulation of the formulated mycelium.

Under field conditions, the mycelium pieces produced as many or more conidia than the fungus on cadavers (experiment 2 $F_{[1,8]} = 48.3$, $P < 0.001$; experiment 5 $F_{[1,6]} = 3.7$, $P = 0.103$) (Table 5). However, when compared on a dry-weight basis, conidial production at 13.5 °C (experiment 5) and 17.1 °C (experiment 2) was 19.4 ($F_{[1,6]} = 74.6$; $P < 0.001$) and 3.0 ($F_{[1,8]} = 31.9$; $P < 0.001$) times greater, respectively, on the fungus-killed hosts than on the formulated mycelium. On the basis of hymenium surface area, the differences in conidial production by the two fungi were less pronounced or absent. At 13.5 °C, fungus on cadavers produced significantly more conidia than formulated mycelium ($F_{[1,6]} = 26.4$; $P = 0.002$), but conidial production per unit area of hymenium was only 5.6-fold greater on cadavers. There was no significant difference in sporulation per unit area between the fungi at 17.1 °C ($F_{[1,8]} = 1.06$; $P = 0.337$).

4. Discussion

4.1. Temporal patterns of sporulation

A study of sporulation of *Z. radicans* isolate 1590 on fifth-instar cadavers of *E. kraemeri* under constant laboratory conditions (100% RH, 5–25 °C) was conducted by Leite et al. (1996b). Regression of the median sporulation times in this case indicated a base temperature of 1.0 °C and a thermal constant of 416.8 DH ($Y = -0.0023 + 0.0024x$; $r^2 = 0.956$) (S.P. Wraight, unpublished). This value is 93 DH lower than the value of 510.2 DH estimated in the present study for 50% sporulation of the dried-mycelium formulation. The initiation of sporulation from a dormant (dry and frozen) state compared to initiation from fresh cadavers might

Table 4
Zoophthora radicans conidia production from a dry-mycelium formulation versus naturally infected *E. kraemeri* on cowpea foliage in a field near Goiânia, Goiás, Brazil, 1987

Night of	Conidia $\times 10^3$ produced per leafhopper cadaver or formulation piece [a] (number of substrates sporulated/monitored)							Temperature (°C) mean (range) while foliage wet	Hours foliage wet	Degree hours under wet conditions
	First-instars	Second-instars	Third-instars	Fourth-instars	Fifth-instars	Adults	Formulation pieces			
18–19 June	0 (0/1)	0.5 ± 0.4 (2/3)	2.6 ± 0.8 (5/6)	5.8 ± 5.4 (4/4)	6.6 ± 5.8 (4/6)	13.4 ± 12.4 (4/6)	0.2 (1/6)	12.8 (9.5–20.5)	14.5	160
19–20 June	1.2 (1/1)	2.3 ± 1.5 (3/3)	6.9 ± 3.2 (6/6)	21.3 ± 8.1 (3/3)	23.9 ± 8 (6/6)	13.9 ± 6.4 (5/5)	0.2 ± 0.2 (3/5)	12 (9–19)	13.5	138
20–21 June	0.4 (1/1)	4.1 ± 1.7 (3/3)	6 ± 1.3 (6/6)	12.6 ± 0.2 (3/3)	34.4 ± 9 (6/6)	53.1 ± 16.9 (5/5)	17.3 ± 12.4 (3/3)	12.8 (10–18.5)	14.5	160
21–22 June	0 (0/1)	2.6 ± 2.4 (2/3)	4.9 ± 2.7 (3/5)	7.2 ± 3.5 (3/3)	35.8 ± 10.4 (6/6)	68.5 ± 22 (4/4)	13.6 ± 4.9 (3/3)	13.9 (11–19)	15	182
22–23 June	0 (0/1)	1.6 ± 1.6 (2/3)	0.4 ± 0.3 (2/4)	6.1 ± 2.8 (3/3)	15 ± 5.1 (5/5)	78.2 ± 32.4 (3/3)	24.5 ± 8.2 (3/3)	14.3 (11.5–19.5)	14.5	181
23–24 June	—	0.4 ± 0.4 (1/3)	0 (0/4)	0.3 ± 0.1 (3/3)	0.8 ± 0.4 (5/5)	36.8 ± 15.8 (3/3)	12.4 ± 3.1 (3/3)	13.4 (10.5–17)	13.5	157
24–25 June	—	0 (0/3)	0 (0/4)	0 (0/3)	0 (0/5)	9.6 ± 5.1 (2/3)	0.4 ± 0.4 (3/3)	15.2 (13.5–17)	13	174
25–26 June	—	0 (0/3)	0 (0/4)	0 (0/3)	0 (0/5)	0.5 ± 0.3 (2/3)	0.01 ± 0.01 (1/3)	13.2 (12–15.5)	13	148
Total	1.6	11.5	20.8	53.3	116.5	274	68.6			
Total from persistent substrates[b]		11.5 ± 4.6 ($n = 3$)	19.2 ± 7 ($n = 5$)	47.9 ± 4.2 ($n = 3$)	123.2 ± 15.7 ($n = 5$)	284 ± 5.6 ($n = 3$)	68.6 ± 20.9 ($n = 3$)			

[a] Mean number of conidia $\times 10^3$ produced per cadaver or piece ($2 \times 2 \times 0.5$ mm) of dried-mycelium formulation ± standard error.
[b] Total conidia $\times 10^3$ (mean ± standard error) produced from leafhopper cadavers or formulation pieces that remained attached to the leaf surface until completion of the sporulation cycle.

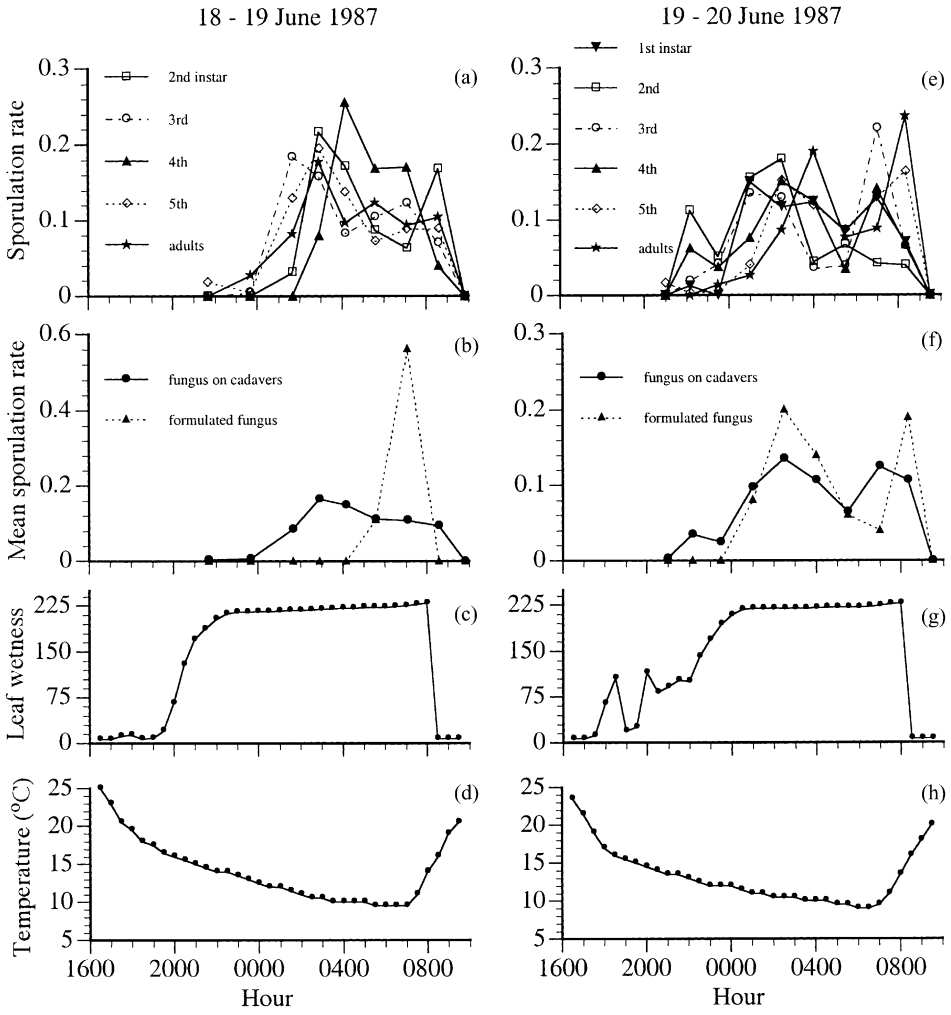

Fig. 8. (a and e) Patterns of *Z. radicans* sporulation from naturally infected second-, third-, fourth-, and fifth-instar and adult leafhopper cadavers over two consecutive nights with temperature and leaf wetness data; (b and f) patterns of sporulation from formulated mycelium versus fungus on naturally infected leafhopper cadavers (mean pattern from all instars); rate expressed as proportion of total conidia/h.

account for this difference. A delay in initial sporulation of formulated fungus compared to fungus on cadavers was evident in the field tests. On night one of the first bean field experiment, the formulation initiated sporulation 6 h after hydration (5.5 h after dewset), whereas the fungus on the freshly killed hosts began producing conidia within 4.5 h. On night two, after the formulated and naturally occurring fungi had dried to presumably similar moisture contents, sporulation of both began 2 h postdewset. On the first night of the second bean field experiment, the fungus on cadavers discharged conidia within only 2 h after dewset, while the formulation required 5 h. Again, on the second night, both fungi produced conidia within 2 h after dewset.

Generally, however, the differences in time required for initial sporulation of the naturally occurring versus formulated fungus on the first night of the field studies was not sufficient to account for the entire 93 DH difference. A more likely explanation relates to the observations of generally faster sporulation of the fungus on cadavers compared to the formulated mycelium (Tables 2–4). The results summarized in Table 2, for example, show that the fungus on fifth-instar cadavers produced 98% of total conidia within two nights, whereas the mycelium pieces produced only 62%.

The more rapid sporulation of the naturally occurring fungus is also reflected in the consistent observation of this fungus reaching peak rates of sporulation earlier than the formulated fungus. This could also be related to significantly different water and time requirements for full rehydration and sporulation of the small amounts of mycelium in the cadavers versus the large masses of mycelium comprising the formulation pieces. On the other hand, the fact that postpeak declines in sporulation (especially of the naturally occurring fungus) often occurred under increasing or high, stable levels of wetness strongly indicates a rhythmic control factor other than nightly moisture levels. These observations are in

Table 5
Zoophthora radicans conidia production from a dried-mycelium formulation versus naturally infected *E. kraemeri* expressed on a substrate, dry weight, and surface area basis

Experiment (crop)	Mean temperature[c] (range)	Conidia $\times 10^3$ produced by fungus on leafhopper cadavers[a]				Conidia $\times 10^3$ produced by dry-mycelium formulation[b]			
		Nights of sporulation[d] (degree hours)	Conidia/ cadaver	Conidia/ mg	Conidia/ mm^2	Nights of sporulation[d] (degree hours)	Conidia/ piece	Conidia/ mg	Conidia/ mm^2
1 (beans)	17.2 (14–23)	5 (700)	117.1 ($n = 2$)	1027.2	58.6	5 (700)	158.9 ($n = 40$)	123.2	25.4
2 (beans)	17.1 (14–21)	4 (695)	99.7 ($n = 6$)	874.6	49.9	5 (908)	371.8 ($n = 4$)	288.2	59.5
4 (cowpeas)	18 (14.5–24)	—	—	—	—	8 (1213)	132.9 ($n = 21$)	108	21.3
5 (cowpeas)	13.5 (9–20.5)	6 (978)	123.2 ($n = 5$)	1080.7	61.6	7 (1152)	68.6 ($n = 3$)	55.8	11

[a] Experiments 2 and 5: conidia produced on fifth-instar cadavers (mean dry weight, 0.114 mg). Experiment 1: conidia produced on 1 fifth- and 1 adult cadaver (mean weight estimated at 0.114 mg); total conidia production underestimated due to storm destruction after five nights.

[b] Conidia produced by 2×2 mm pieces of dry-mycelium formulation, with a mean dry weight of 1.29 and 1.23 mg for isolates 789 (experiments 1 and 2) and 1590 (experiments 3 and 4), respectively. Fungal hymenium, when fully developed, measured approximately 2 and 6.25 mm^2 on leafhopper cadavers and mycelium pieces, respectively.

[c] Mean hourly temperature during experiment (during wet conditions while fungus retained significant sporulation capacity ($> 0.1 \times 10^3$ conidia/substrate/night)).

[d] Number of nights conidia were produced in significant numbers; number in parentheses indicates total degree hours accumulated during wet conditions.

agreement with the findings of Yamamoto and Aoki (1983), who concluded that circadian periodicity of *Z. radicans* sporulation was exogenously conditioned by light, with the peak discharge during the night being the result of a delayed light-positive reaction. Under a laboratory light regime of 12L:12D, peak rates of sporulation were observed during the dark period, with sporulation falling off soon after initiation of the photophase. Daylength was also approximately 12 h in the field during our experiments.

The fact that sporulation of the mycelium pieces reached highest levels after dawn suggests a disruption of the fungus' biological clock. Laboratory culture under artificial and irregular light regimes combined with storage under dark conditions may have been important factors. If that were the case, one would expect to see evidence of a resetting of the biological clock after the first day in the field (evidenced by a shift in time of peak sporulation on night two). Such a shift is suggested by the data from three of four experiments (Figs. 5–8). Unfortunately, no hourly sampling was conducted after night two. Sampling during later sporulation episodes might have revealed increasingly similar sporulation patterns between the naturally occurring and formulated fungi. It could prove valuable for mycoinsecticide producers to investigate this further, as benefits might be realized in terms of microbial control if the fungus could be conditioned to sporulate at peak rates earlier in the night.

Alternatively, the peaks in sporulation of the formulated mycelium appear directly related to the post-dawn increases in temperature (Figs. 6–8). Interestingly, the conidia production data from the field experiments (Table 5) suggest that the mycelium formulation may be more sensitive to low temperatures than the fungus on leafhopper cadavers. This is suggested also by data from laboratory studies. In studies of temperature-dependent sporulation of *Z. radicans* on leafhopper cadavers, Leite et al. (1996b) observed that 2.1 times more conidia were produced at the most favorable versus least favorable temperatures tested (20 versus 5 °C). In contrast, the dried-mycelium formulation monitored in this study (Table 1) produced 4.4 times more conidia at 20 than at 5 °C. Also, analysis of the median sporulation rates reported by Leite et al. (1996b) indicates a sporulation threshold of 1.0 °C compared to the estimate of 1.8 °C derived from the formulated mycelium (Fig. 4).

The entomophthoralean fungi have variable moisture requirements for conidiogenesis. *Entomophthora muscae* (Cohn) Fresenius is capable of releasing conidia in very dry atmospheres (Kramer, 1981), and Hajek et al. (1990) recently reported limited sporulation of *Entomophaga maimaiga* Humber, Soper and Shimazu under relative humidity (RH) conditions as low as 70%. Various studies have indicated, however, that species of the genus *Zoophthora* require moisture-saturated or nearly saturated conditions for sporulation (Galaini-Wraight et al., 1991; Glare et al., 1986; Millstein et al., 1983). Our observations of *Z. radicans* sporulation commencing shortly after dewset and ceasing after drying of the foliage are in accord with these studies. Dew formation (100% RH) was observed soon after sunset on all of our study nights, and we therefore did not attempt to characterize the role of moisture measured as RH in the

natural *Z. radicans* sporulation process. Nevertheless, increases in RH precede detection of leafwetness, and high humidity during the early evening hours initiates the hydration process that culminates in conidia production after dewset. Also, the level of RH (in combination with temperature and wind velocity) during the daylight hours determines the water content of the fungus lying dormant in the cadavers on the leaf surface between the nightly sporulation episodes. This would affect the time required for the fungus to fully rehydrate when exposed to humid air or free water in the form of dew. Because infection of the insect host may be limited to relatively short daily periods of moist conditions such as described by Galaini-Wraight et al. (1992) and the host penetration process is rapid (Wraight et al., 1990), even a small decrease in the time to initiation of sporulation could have epizootiological significance.

4.2. Conidial production

Sporulation of *Z. radicans* appears as great under fluctuating field conditions as under constant optimal laboratory conditions. For example, mycelium of isolate 789 produced 1.37×10^5 conidia/mg at constant 20 °C in the laboratory compared to $1.23–2.88 \times 10^5$ conidia/mg at an average temperature of 17 °C in the field (cf. Tables 1 and 5). Also, *Z. radicans* isolate 1590 on adult cadavers incubated at 20 °C in the laboratory produced only 2.6×10^4 conidia (Leite et al., 1996b), whereas *Z. radicans* in field experiment five produced a total of 2.8×10^5 conidia/adult cadaver (Table 4). The reason for this substantial difference is not known. Milner and Lutton (1983) reported similar high levels of *Z. radicans* conidia production from spotted alfalfa aphids in the laboratory at 25 °C (mean 3.7×10^5 conidia/cadaver). The aphid isolate used (originally from Israel) had a slightly higher temperature optimum for sporulation (≈25 °C) than isolate 789. Although environmental conditions were clearly favorable to *Z. radicans* during the transition period from the rainy to dry season in central Brazil, with consistently high-overnight moisture levels (Galaini-Wraight et al., 1991), this level of conidia production in the field is nevertheless only possible through the fungus' capacity to undergo multiple daily sporulation/desiccation cycles. The cadavers appeared completely dry during the warm afternoon hours (mean maximum temperatures during May in this study were 29–30 °C). The dried and shrunken early-instar cadavers were scarcely noticeable under these conditions. The nightly transformation of the fungus was remarkable.

Few other studies have quantified conidia production from dried mycelium of *Z. radicans*. Soper (1985) reported initial sporulation at 16, 10, 6, and 4 h at 5, 10, 15, and 20 °C, respectively; these times are similar to the observations of the present study (see results). Pell et al. (1998) recorded sporulation from freshly dried mycelium preparations of a diamondback moth isolate of *Z. radicans* from Malaysia (isolate NW250). Mean production was 6.4×10^4 conidia/cm^2 over a period of approximately 90 h at 20 °C. This production level is low compared to the dried-mycelium formulation used in the present study (Table 5). Li et al. (1993) produced a dried-mycelium preparation of isolate NW250 which yielded approximately 4×10^4 conidia/mg at room temperature. This material, however, proved highly unstable, losing >95% of its conidial-production capacity after storage for 80 days at 4 °C, or 36 days at −20 °C. Milling of the fungus to granules <1 mm in size had an equally detrimental effect; milled mycelium produced $<10^3$ conidia/mg.

This experience with poor cold storage stability of *Z. radicans* dried-mycelium is in sharp contrast to our findings. The dried-mycelium formulation applied in the June 1987 field experiments had been stored at −20 °C for >1 year, yet produced $>10^5$ conidia/mg under field conditions. Also, the milling process used in the present study had no comparable detrimental effect on sporulation. The milled granules of isolate 789 produced $>10^5$ conidia/mg at 20 °C (Table 5). The instability of the dry mycelium produced by Li et al. (1993) compared to that used in the present study may be attributable to differences between the *Z. radicans* strains; however, there were also potentially important differences in production protocols. We produced dry mycelium strictly according to the McCabe and Soper (1985) process, whereas Li et al. (1993) made significant changes in the protocol, including a prolonged room-temperature incubation (24 h) followed by desiccation at 4 °C.

There are few published estimates of low-temperature thresholds for *Z. radicans* development. Milner and Lutton (1983) estimated thresholds for radial hyphal growth and pathogenesis of 6.7 and 3.7 °C, respectively. Sawyer (1929) reported that hyphal growth was arrested at 8 °C. The data presented in Fig. 4 indicated a substantially lower threshold for conidial production (1.8 °C). The usefulness of thresholds determined from constant temperature studies under optimal moisture conditions (on culture media) in the laboratory, however, must be assessed with caution. Most importantly, their significance with respect to events in the field is dependent upon the high-moisture requirements of *Z. radicans*. Extended lengths of time are required for initiation of sporulation at low temperatures (Table 1), and conidia production in the field at temperatures below 15 °C is therefore severely limited by the available time under wet conditions. Thus, a mean of only 30 conidia was produced during the night of 6/7 June even though highly favorable moisture conditions existed for 12.5 h and the temperature (mean 7.3 °C) was well above the estimated threshold. This phenomenon suggests that the laboratory constant temperature threshold of 1.8 °C is

an underestimate in terms of disease development or epizootic potential under normal field conditions. It appears unlikely from the Table 2 data that sporulation by the mycelium pieces would occur at a mean temperature below 5–6 °C. In considering the overall effects of low temperature on epizootic potential, however, it must be noted that low temperatures induce *Z. radicans* ballistoconidia to produce capilliconidia (Galaini-Wraight et al., 1992; Van Roermund et al., 1984), and capilliconidia are more capable of surviving daytime low-humidity conditions than ballistoconidia (Griggs et al., 1999; Uziel and Kenneth, 1991; Uziel and Shtienberg, 1993).

4.3. Epizootic potential of the dried-mycelium formulation

The *Z. radicans* dry-mycelium mass production and formulation processes have not been developed to commercial scale, and the current requirement for production, processing, cold storage, and delivery are severe constraints to economical use of this material as a mycoinsecticide for broadcast (inundative) microbial control applications. Strategies for use of mycelial preparations have, therefore, focused on augmentative releases to initiate epizootics in areas where the fungus is either not active (Pell and Wilding, 1994; Wraight et al., 1986; Wraight and Roberts, 1987) or where epizootics of indigenous pathogens do not normally develop in time to prevent crop damage by the target pest (Leite, 1991). In the studies reported by Wraight et al. (1986) and Wraight and Roberts (1987), high-rate applications of mycelium fragments (10 g/m^2) were applied to dew-covered foliage in small 2×2 m plots to establish infection foci from which a fieldwide epizootic was initiated.

On the natural host cadaver, the fungus began to produce conidia within a few hours after dewset, and reached peak levels of sporulation between midnight and 03:00 h. Nightly high-moisture conditions prevailed until approximately 08:00 h, leaving sufficient time during the remainder of the night for the conidia to germinate and complete the infection process (the time required for infection of 10 and 50% of individuals inoculated with a lethal dose was determined by Galaini-Wraight et al. (1992) to be approximately 6 and 14 h at 15 °C, respectively). This is clearly an important factor contributing to the extremely high-epizootic potential of this fungal pathogen.

The formulated mycelium pieces produced equivalent or greater numbers of conidia than the fungus on the fifth-instar cadavers (Table 5), and although sporulation by the formulated fungus peaked, in most cases, after dawn, it commenced within 2–3 h after initial sporulation by the fungus on cadavers and produced rates of conidial production comparable to the naturally occurring fungus by 03:00 h on five of six nights (Figs. 5, 6, and 8). In addition, the 2×2-mm mycelium pieces produced conidia longer (more nights) than the fungus on all host cadavers, except adults, and produced more conidia than the fungus on early mid instar cadavers. These results indicate that the McCabe and Soper (1985) dried-mycelium formulation of *Z. radicans* possesses characteristics that make it exceptionally well suited as an inoculum source for establishment or augmentation of natural epizootics in pest populations and support previously cited reports of field tests in which applications of the dry mycelium successfully initiated or augmented field-wide epizootics in pest populations.

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